Supercoiled circular DNA of an insect granulosis virus

(high-molecular-weight DNA/electron microscopy/physical chemical characterization)

KATHLEEN A. TWEETEN*, LEE A. BULLA, JR.†, AND RICHARD A. CONSIGLI*‡

* Division of Biology, Kansas State University, Manhattan, Kansas 66506; and † U.S. Grain Marketing Research Center, Agricultural Research Service, U.S. Department of Agriculture, Manhattan, Kansas 66502

ABSTRACT   The DNA of the granulosis virus of the Indian meal moth, *Plodia interpunctella*, was characterized by physical chemical and electron microscopic techniques. Twenty-five percent of the DNA extracted from purified virus was isolated as supercoiled circular molecules. The remaining 75% consisted of relaxed circular molecules. These molecular forms were indicated by the production of two radioactive bands during sedimentation of ^{3}H-labeled granulosis virus DNA in alkaline sucrose gradients or in equilibrium density gradients of neutral cesium chloride/propidium iodide. Electron microscopic visualization of the DNA that banded at the higher density in the latter gradients revealed supercoiled structures whereas that of DNA that banded at the lower density demonstrated relaxed circular molecules. The superhelical molecules were converted to relaxed circles by treatment with pancreatic DNase.

The molecular weight of the viral DNA was calculated to be 81×10^6 by sedimentation in neutral sucrose and 78×10^6 by sedimentation in alkaline sucrose. The molecular weight estimated from length measurements in electron micrographs was 76×10^6. The buoyant density of the granulosis virus DNA was 1.703 g/cm^3 and that of its insect host DNA was 1.697 g/cm^3. Equilibrium sedimentation in cesium chloride and thermal denaturation indicated G + C contents of 44% and 39% for the viral and host DNA, respectively.

Granulosis and nuclear polyhedrosis viruses, members of the genus *Baculovirus*, are being considered for use as biological insecticides. Such an alternative to chemical pesticides is especially important in the case of the Indian meal moth, *Plodia interpunctella*. This insect, a serious pest of stored products, is difficult to control because of its resistance to chemicals such as malathion and synergistic pyrethrins (1). Recent studies have demonstrated that a granulosis virus (GV) can effectively control populations of Indian meal moth (2, 3). In our laboratory, we are concerned with the biochemical basis for the insecticidal activity of this virus. Because little is known of the molecular biology of GV, we initiated our studies by characterizing the components of the virus biophysically.

The present report describes the DNA of the granulosis virus of *P. interpunctella*. Others (4–7) have reported that DNA of other GV is a large (80 to 120 × 10^6 dalton) circular duplex. Shvedchikova *et al.* (4) observed cyclic molecules in Kleinschmidt preparations of DNA that was isolated from a GV of the Siberian silkworm, *Dendrolimus sibiricus*. Band sedimentation of the DNA from the GV of the fall armyworm, *Spodoptera frugiperda* (6), and the cabbage looper, *Trichoplusia ni* (7), in alkaline sucrose and in equilibrium density gradients of cesium chloride/ethidium bromide provided further evidence for the circularity of insect GV DNA and indicated that a portion of the molecules were covalently closed in both strands. Using centrifugation techniques, we have separated and recovered both covalently closed and relaxed

circular DNA molecules from the GV of the Indian meal moth. Our electron microscopic observations confirm the structure of the relaxed circular form and substantiate, for the first time, that the configuration of the covalently closed DNA of a GV is superhelical.

MATERIALS AND METHODS

Production and Purification of ^{3}H-Labeled GV. Inclusion bodies were produced in a laboratory colony of Indian meal moth by *per os* infection of early third instar larvae. At 72, 96, and 120 hr after infection, the larvae were injected with 1 μl (0.5 μCi) of [^{3}H]thymidine (71 Ci/mmol, Schwarz/Mann, Orangeburg, NY). Two to 3 days after the final injection, infected larvae were disrupted in a Sorvall Omnimixer and the resulting homogenate was centrifuged at 1000 × g for 30 min. The supernatant was further centrifuged at 16,000 × g for 30 min. The pellet was resuspended and treated with sodium deoxycholate at a final concentration of 1% (vol/vol) for 15 min at room temperature. The inclusion bodies were then pelleted through a 4-ml shelf of 50% (wt/wt) sucrose by centrifugation at 25,000 rpm (SW 27 rotor) for 2 hr. The preparation was resuspended and centrifuged at 20,000 rpm (SW 27 rotor) for 2 hr in a 45–60% (wt/wt) sucrose gradient. The inclusion bodies were recovered from the gradient, washed twice with distilled H$_2$O, and stored at −20°.

Purification of Viral DNA. GV DNA was isolated by treatment of purified viral inclusion bodies with 0.05 M Na$_2$CO$_3$/0.05 M NaCl, pH 10.6, at room temperature for 30 min. The preparation was then incubated at 37° for 1 hr with Sarkosyl at a final concentration of 2% (vol/vol) and centrifuged in either alkaline sucrose or cesium chloride/propidium iodide (CsCl/PI) gradients. Molecular weight and density estimates were made on DNA isolated as described above except that after Sarkosyl treatment the preparation was neutralized with 0.01 M Tris, pH 7.4, and extracted three times with phenol. The DNA was then dialyzed for 3 days against daily changes of 0.01 M Tris/0.01 M EDTA, pH 7.6, and stored at 4°. GV DNA used in thermal denaturation studies was purified by banding in neutral CsCl gradients (initial density, 1.7 g/cm^3) after phenol extraction. Centrifugation was at 40,000 rpm (SW 50.1 rotor) for 36 hr. The DNA was recovered from the gradient, dialyzed for 24 hr against 0.1-strength 0.15 M NaCl/0.015 M sodium citrate (SSC), and stored at 4°.

Purification of Indian Meal Moth DNA. Larval cell nuclei were isolated from late fourth instar insects. Five hundred larvae were ground in 100 ml of cold SSC in a Pyrex hand homogenizer, filtered through gauze, and centrifuged at 5000 × g for 30 min. The pellet was resuspended in 10 ml of SSC containing 1% Triton (vol/vol) and held for 10 min at room tem-
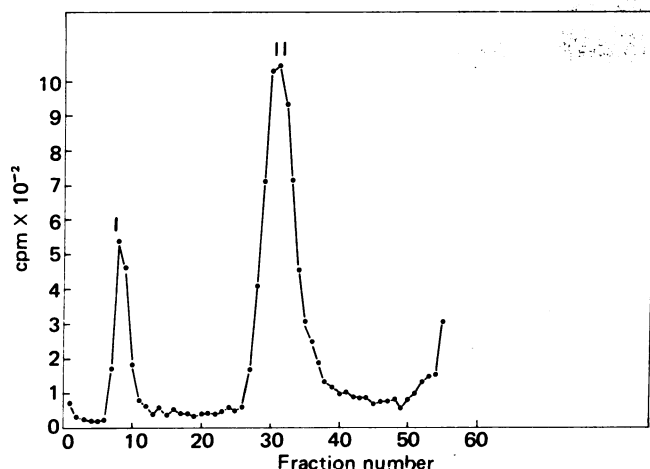
FIG. 1. Fractionation of ^{3}H-labeled GV DNA into fast-sedimenting (I), 20.9%, and slow-sedimenting (II), 79.1%, forms in alkaline sucrose gradient (2–40%, wt/vol, in 1.0 M NaCl/0.3 M NaOH/10 mM EDTA). Centrifugation was at 40,000 rpm (SW 50.1 rotor) for 1 hr at 10°. Fractions were collected and acid precipitated, and radioactivity was measured.
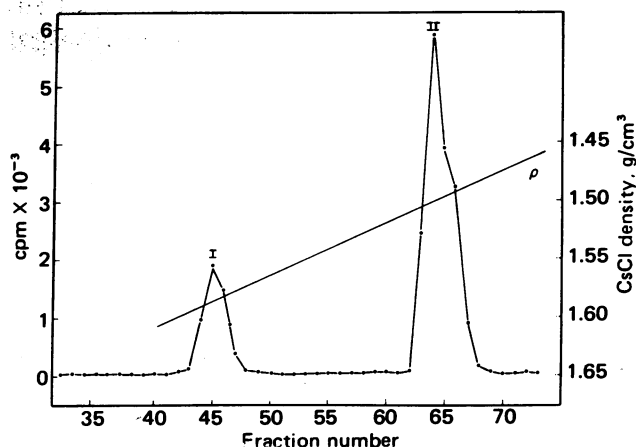

FIG. 2. Separation of form I GV DNA (band of higher density) from form II (band of lower density) by equilibrium centrifugation in CsCl/PI gradient. Form I = 23.9%; form II = 76.1%. Gradient had initial density of 1.58 g/cm^3, contained 10 mM EDTA and PI (300 μg/ml), and was centrifuged at 40,000 rpm (SW 50.1 rotor) for 36 hr at 10°. Fractions were collected, radioactivity was measured, and refractive index was determined.

perature. Nuclei were pelleted by centrifugation at 5000 × *g* for 30 min and were rinsed twice with 5-ml portions of SSC. DNA was then isolated from the nuclei as described (8).

Sucrose Gradients. Alkaline sucrose gradients (2–40%, wt/vol) were prepared in 1.0 M NaCl/0.3 M NaOH/10 mM EDTA. Samples consisting of 2–5 μg of viral DNA in 0.3 M NaOH (final concentration) were layered on the gradients. Centrifugation was at 40,000 rpm (SW 50.1 rotor) for 1 hr at 10°. Fractions (9 drops) were collected directly onto Whatman 3 MM filter paper discs. The filters were dried, fixed in cold 10% (vol/vol) trichloroacetic acid, washed in acetone, and assayed for radioactivity in nonaqueous scintillation fluid in a Beckman LS-233 scintillation counter. Neutral sucrose gradients (5–20%, wt/vol) were made in 1.0 M NaCl/0.01 M Tris, pH 8.0 (9). Samples consisting of 2–5 μg of GV DNA were layered on the gradients and centrifuged at 40,000 rpm (SW 50.1 rotor) for 140 min at 10°. Fractions (8 drops) were collected in Triton scintillation mixture and radioactivity was measured. Molecular weight determinations were made according to Studier (9) and Friefelder (10) with bacteriophage T4 and T7 DNAs as markers. In all calculations, molecular weights of 108 × 10^6 and 25 × 10^6 were used for T4 DNA and T7 DNA, respectively (10).

Cesium Chloride Gradients. CsCl/PI gradients were prepared according to Radloff *et al.* (11) except that they contained 10 mM EDTA and PI (300 μg/ml) instead of ethidium bromide (12, 13). Centrifugation was at 40,000 rpm (SW 50.1 rotor) for 36 hr at 10°. Fractions (6 drops) were collected, radioactivity was determined in Triton scintillation fluid, and refractive index was measured in a Bausch and Lomb refractometer. PI was removed by extracting the DNA-containing fractions twice with CsCl-saturated isopropanol.

Buoyant density determinations were made in neutral CsCl gradients (initial density, 1.7 g/cm^3) centrifuged to equilibrium at 40,000 rpm (SW 50.1 rotor) for 48 hr at 10°. ^{32}P-Labeled T7 DNA (14) was incorporated as a marker into gradients containing either 2–5 μg of ^{3}H-labeled GV DNA or 40–80 μg of host DNA. Fractions (10 drops) were collected and their radioactivity and refractive index were determined. The band of host DNA was identified in a Zeiss M4 QIII spectrophotometer by absorbance at 260 nm. The G + C content of the DNA was calculated as described (15).

Thermal Denaturation. Denaturation profiles of GV DNA and Indian meal moth DNA were obtained on a Cary 16 spectrophotometer in 0.1-strength SSC. Midpoints were determined according to Mandel *et al.* (16) with T7 DNA (14) as a standard.

Electron Microscopy. GV DNA was prepared for visualization by the methods of Kleinschmidt (17) and was examined in a Philips EM 201. An electronic graphic calculator (Numonics Corp.) and a map measurer were used for length measurements. Molecular weights were determined by comparing the length of circular GV DNA (40 molecules measured) with that of either polyoma DNA (18) or λ DNA (19) molecules observed in the same field.

RESULTS

Centrifugation of radioactively labeled GV DNA in alkaline sucrose gradients resolved two species (Fig. 1). About 21% of the DNA was in the fast sedimenting band (form I) and 79% was in the slower sedimenting band (form II). Similar proportions of DNA were observed in the two bands produced by equilibrium density gradient centrifugation of purified GV DNA in CsCl/PI (Fig. 2). The DNA that banded at a density of 1.585 g/cm^3 (form I) accounted for 24% of the total radioactive GV DNA and the DNA banding at the lower density (1.500 g/cm^3; form II) contained 76%.

The physical nature of the two forms of GV DNA separated by CsCl/PI equilibrium centrifugation was examined by electron microscopy. Electron micrographs of form I DNA (Fig. 3 *a–c*) revealed that greater than 40% of the molecules had superhelical configurations. Supercoiling of the GV DNA molecules was so extensive that the number of superhelical turns per molecule could not be estimated from the micrographs. All of the remaining molecules seen in these Kleinschmidt preparations were relaxed circular structures (Fig. 3*b*). These relaxed circles probably were caused by nicking of superhelical DNA during its recovery from the CsCl/PI gradient and subsequent handling for electron microscopy. After such treatment, only 40–50% of the supercoiled DNA banded a second time as form I in CsCl/PI density gradients (results not shown). Electron micrographs of GV DNA isolated as form II DNA showed a predominance of relaxed circular molecules (Fig. 3 *d* and *e*). No supercoiled molecules were observed in form II preparations and only 3–5% of the DNA observed was linear.
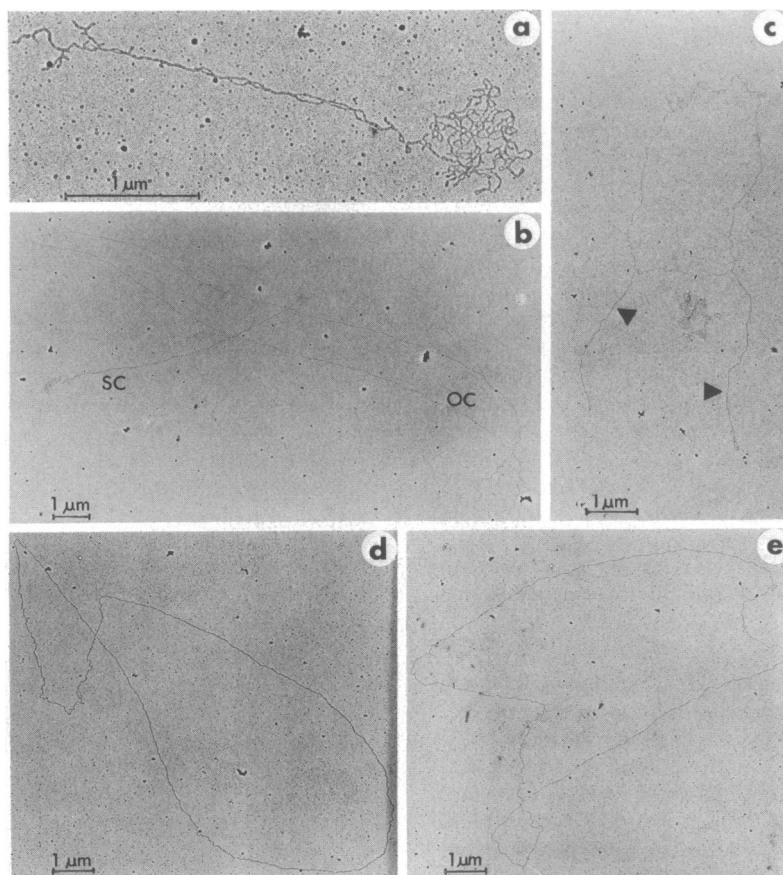
FIG. 3.   Electron micrographs of GV DNA recovered from CsCl/PI gradient. (*a*, *b*, and *c*) Supercoiled molecules typical of DNA that banded at the higher density. The superhelical (SC) structure in *b* is across a relaxed open circular (OC) molecule and that in *c* is only partially supercoiled (arrows). (*d* and *e*) Relaxed circular molecules characteristic of the DNA that banded at the lower density.

Superhelical GV DNA molecules were converted to relaxed circles by DNase treatment. Samples of purified GV DNA incubated with or without pancreatic DNase were analyzed by centrifugation to equilibrium in CsCl/PI gradients (Fig. 4). Whereas 26% of the DNA in untreated samples banded as form I, 100% of the DNA in the DNase-treated samples was recovered as form II. Similar results were obtained when supercoiled DNA that had been purified in a CsCl/PI gradient was treated with DNase and then recentrifuged in CsCl/PI gradients (results not shown). Superhelical GV DNA was also converted to form II by shearing because molecules that had been exposed to mixing (tube inverted 12 times/min for 5 min) during phenol extraction banded only as relaxed circular molecules in CsCl/PI gradients.

Centrifugation of form II GV DNA in neutral sucrose gradients resulted in a band of DNA sedimenting between T7 DNA and T4 DNA (Fig. 5 *upper*). To minimize the effect of the cyclic shape of the DNA on its rate of sedimentation, the gradients were centrifuged at 40,000 rpm. At this speed, circular GV DNA sediments only 1–3% faster than its corresponding linear form (20). When compared to the bacteriophage DNAs, GV DNA had a sedimentation coefficient of 55–56 S. This value corresponded to molecular weight estimates of 80×10^6 and 83×10^6 with respect to T7 DNA and T4 DNA, respectively. Relaxed circular GV DNA, sedimented in alkaline sucrose gradients (Fig. 5 *lower*), exhibited a sedimentation coefficient of 62 S with respect to T4 DNA. A molecular weight of 78×10^6, based on this S value, was calculated for GV DNA.

The molecular weight of GV DNA was also estimated from electron micrographs of the DNA. Measurements made from photographic fields in which both open circular GV DNA and open circular polyoma DNA were spread (Fig. 6) indicated that GV DNA molecules were homogeneous in size and had an average length of 37 μm. An average length of 36.8 μm was obtained for GV DNA from measurements made of fields con-
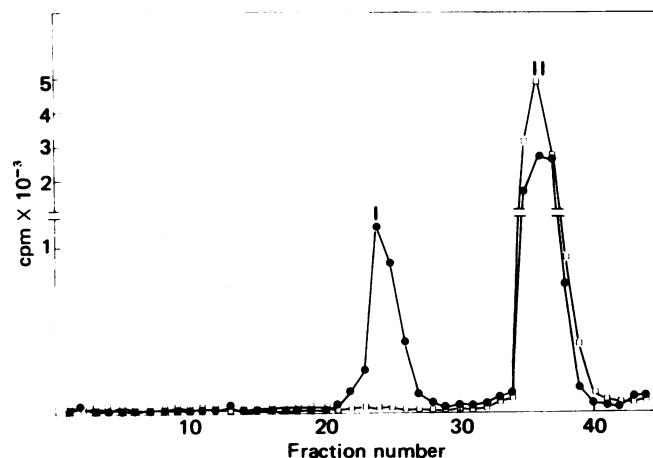

FIG. 4.   Conversion of supercoiled GV DNA to form II by treatment with DNase. GV DNA (10 μg) was incubated with pancreatic DNase (0.5 μg/ml) for 10 min at 37° in presence of 10 mM MgCl₂/bovine serum albumin (100 μg/ml). Centrifugation of untreated (●) and treated (□) DNA was as described in legend to Fig. 2. Without DNase: form I, 26.4%; form II, 73.6%. With DNase: form I, 0%; form II, 100.0%.

Microbiology: Tweeten et al.
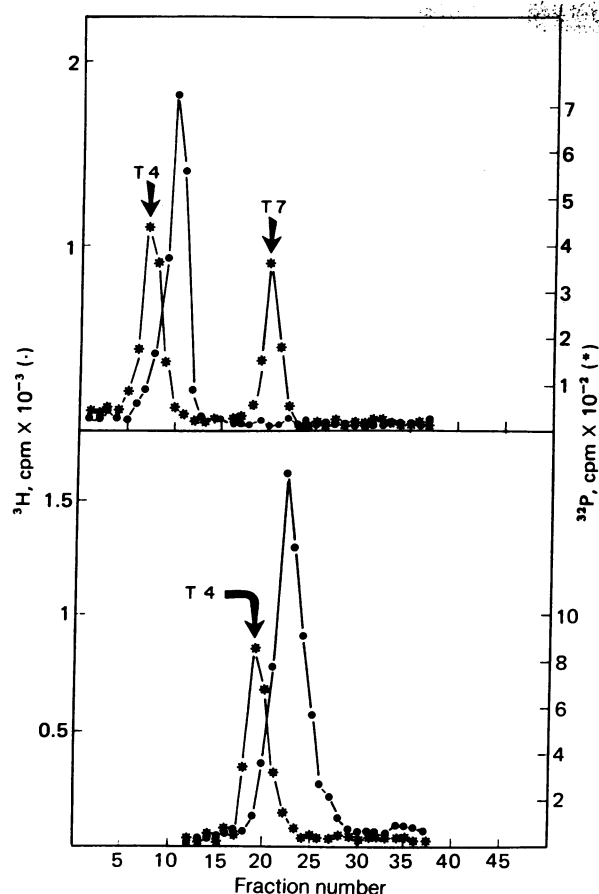
Proc. Natl. Acad. Sci. USA 74 (1977)   3577

FIG. 5. (*Upper*) Sedimentation of relaxed circular GV DNA (●) in neutral sucrose gradient (5–20%, wt/vol, in 1.0 M NaCl/0.01 M Tris, pH 8.0) containing ^{32}P-labeled T4 and T7 DNA (*) as markers. Centrifugation was at 40,000 rpm (SW 50.1 rotor) for 140 min at 10°. Fractions were collected and radioactivity was measured. Molecular weight was estimated as described (9, 10). (*Lower*) Sedimentation of relaxed circular GV DNA (●) in alkaline sucrose gradient. Conditions were as described in legend to Fig. 1. Molecular weight was calculated according to Studier (10) with respect to T4 DNA (*).

Table 1. Determination of G + C content of DNA isolated from GV and fourth instar Indian meal moth larvae

DNA	Density in CsCl, g/cm^3	G + C content based on density, %	t_m, °C*	G + C content based on t_m, %
T7	1.710	50.0	73.2	50.0
GV	1.703	43.8	70.8	44.4
Host	1.697	37.7	69.0	40.0

* t_m = melting temperature.

taining λ DNA as a marker. An average molecular weight of 76.4 × 10^6 was calculated for the GV DNA from these lengths.

Equilibrium centrifugation of GV DNA or insect host DNA in CsCl with T7 marker DNA indicated a buoyant density of 1.703 g/cm^3 for GV DNA and 1.697 g/cm^3 for Indian meal moth DNA. The G + C percentages calculated from these density measurements were 43.8% and 37.7%, respectively. The base compositions of the DNAs were also analyzed by thermal denaturation studies. The melting temperature determined for GV DNA with respect to T7 DNA was 70.8° whereas that of the host DNA was 69.0°. The G + C contents computed from these values were 44.4% for GV DNA and 40% for Indian meal moth DNA (Table 1).

DISCUSSION

The sedimentation in alkaline sucrose (Fig. 1), the banding pattern in CsCl/PI (Fig. 2), and susceptibility to DNase treatment (Fig. 4) exhibited by the DNA of the GV of *P. interpunctella* are characteristic of circular duplex DNA having supercoiled and relaxed circular forms (21). Electron microscopic examination confirmed these physical chemical properties by revealing that purified GV DNA consists of cyclic molecules having either a superhelical or relaxed configuration (Fig. 3).

Although 25% of the DNA isolated and recovered from GV was supercoiled, it is very likely that a higher percentage of the DNA molecules have a supercoiled configuration within the viral nucleocapsid. The large size of the DNA renders it very susceptible to shear and, consequently, many single-strand breaks may have been introduced into superhelical molecules during their isolation and purification. This was especially evident when purification of the DNA included phenol extraction; we observed a complete loss of form I DNA if extensive mixing was done during such extraction. This may explain why Kok et al. (22) failed to observe superhelical structures in Kleinschmidt preparations of nuclear polyhedrosis virus DNA that had been purified by phenol extraction.

The preponderance of open circular molecules in purified preparations of form II DNA (Fig. 3 d and e) confirms other observations (4, 5, 7) that the DNA naturally present in GV is circular. Only 2–3% of the DNA in our preparations was linear and probably resulted from double-strand scissions of the circular molecules. Size homogeneity of the relaxed circular molecules was demonstrated by electron microscopy (Fig. 6) and by sedimentation in sucrose gradients (Fig. 5). The electron micrographic estimate of molecular weight, 76.4 × 10^6, corresponded to the molecular weight of 80 × 10^6 determined from sedimentation of the DNA in alkaline and neutral sucrose gradients (Fig. 5). These estimates indicate that the DNA of the *P. interpunctella* GV is one of the largest circular forms of duplex DNA known. Only the cyclic DNAs of the GV and nuclear polyhedrosis virus of *T. ni* and *S. frugiperda*, whose molecular weights were estimated by sedimentation to be 91 to 100 × 10^6 (7, 20), are larger. The base composition of *P. interpunctella* GV DNA differs from that of other characterized GV DNAs. The G + C contents of DNA of the *T. ni*, *P. interpunctella*, and *S. frugiperda* GVs are 37.5%, 44%, and 50%, respectively. Interestingly, the DNA base compositions of the hosts of these viruses are very similar. We calculated an average G + C content of 39% for *P. interpunctella* DNA whereas the G + C content of *S. frugiperda* DNA is 38% and that of *T. ni* DNA is 36% (7).

The size homogeneity of the DNA molecules isolated by us and others (7) from GVs may be characteristic of all GV DNA. In contrast, the DNA molecules isolated from many nuclear polyhedrosis viruses are heterogeneous in size (22–24). Although this heterogeneity may have been generated by the purification methods used (20), electron microscopy has revealed circular molecules of significantly different lengths in nuclear polyhedrosis virus DNA preparations (22, 23). These observations, along with evidence that the low-molecular-weight molecules in these preparations are infectious (25–27), have led to the hypothesis that the DNA contained in nuclear polyhedrosis viruses is polygenomic. The infectivity of GV DNA has not been investigated, so it is not known whether the circular molecules of *P. interpunctella* GV DNA are monomers or oligomers.
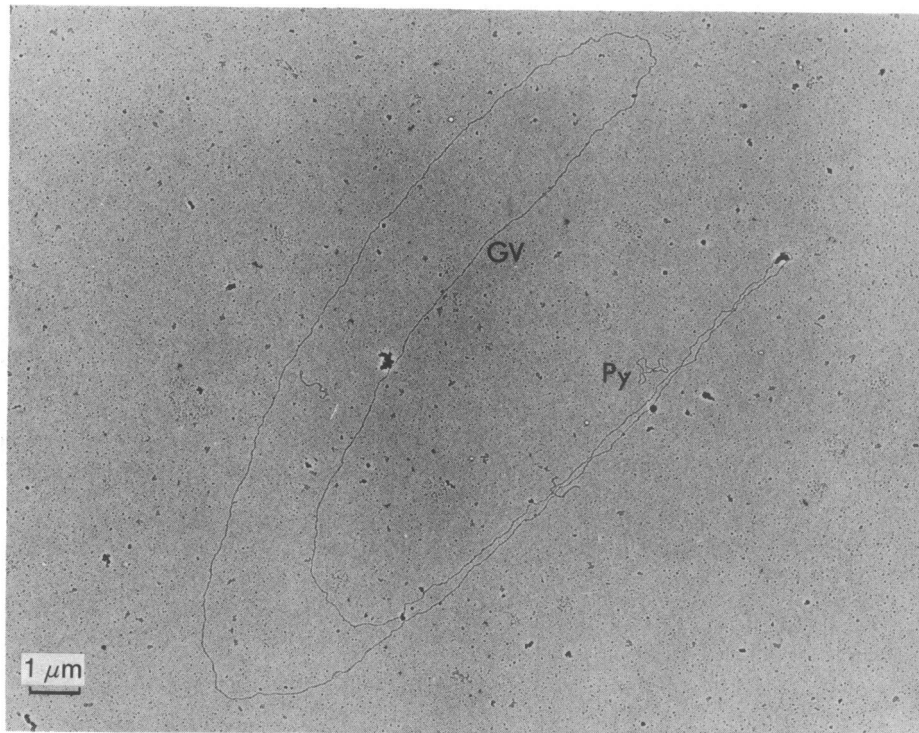
FIG. 6.   Electron micrograph from which the length of granulosis virus (GV) DNA was measured and compared to that of polyoma virus (Py) DNA.

Because the preparations of GV DNA are homogeneous in size, the investigation of their biological activity and genomic complexity may help resolve the question concerning the size of the *Baculovirus* genome.

The physicochemical and structural analyses of the GV DNA described here have substantiated reports (4, 6, 21, 24) of the natural occurrence of supercoiled circular duplex DNA of high molecular weight in insect baculoviruses. Future studies on the GV DNA in insect tissue cultures will provide information on the molecular biology of these relatively uncharacterized viruses and also could give insight into the mode of replication and packaging of a high-molecular-weight circular DNA and on the interaction of a genome of high complexity with its eukaryotic host.

1. Zettler, J. L., McDonald, L. L., Redlinger, L. M. & Jones, R. D. (1973) *J. Econ. Entomol.* **66**, 1049–1050.
2. Hunter, D. K., Collier, S. J. & Hoffmann, D. F. (1973) *J. Invertebr. Pathol.* **22**, 481.
3. McGaughey, Wm. H. (1975) *J. Econ. Entomol.* **68**, 346–348.
4. Shvedchikova, N. G. & Tarasevich, L. M. (1968) *13th Int. Congr. Entomol. (Moscow)*, Abstr. Papers, p. 244.
5. Shvedchikova, N. G. & Tarasevich, L. M. (1971) *J. Invertebr. Pathol.* **18**, 25–32.
6. Summers, M. D. & Anderson, D. L. (1972) *J. Virol.* **9**, 710–713.
7. Summers, M. D. & Anderson, D. L. (1972) *Virology* **50**, 459–471.
8. Laird, C. D. & McCarthy, B. J. (1968) *Genetics* **60**, 303–322.
9. Studier, F. W. (1964) *J. Mol. Biol.* **11**, 373–389.
10. Freifelder, D. (1970) *J. Mol. Biol.* **54**, 567–577.
11. Radloff, R., Bauer, W. & Vinograd, J.(1967) *Proc. Natl. Acad. Sci. USA* **57**, 1514–1521.
12. Hudson, B., Upholt, W. B., Devinny, J. & Vinograd, J. (1969) *Proc. Natl. Acad. Sci. USA* **62**, 813–820.
13. Fukuda, A. (1976) *J. Biochem.* **80**, 253–258.
14. Szybalski, W. (1968) in *Methods in Enzymology*, eds. Grossman, L. & Moldave, K. (Academic Press, New York), Vol. 12B, pp. 330–360.
15. Schildkraut, C. L., Marmur, J. & Doty, P. (1962) *J. Mol. Biol.* **4**, 430–443.
16. Mandel, M. & Marmur, J. (1968) in *Methods in Enzymology*, eds. Grossman, L. & Moldave, K. (Academic Press, New York), Vol. 12B, pp. 195–206.
17. Kleinschmidt, A. K. (1968) in *Methods in Enzymology*, eds. Grossman, L. & Moldave, K. (Academic Press, New York), Vol. 12B, pp. 361–377.
18. Weil, R. & Vinograd, J. (1963) *Proc. Natl. Acad. Sci. USA* **50**, 730–738.
19. Young, E. T., II & Sinsheimer, R. L. (1967) *J. Mol. Biol.* **30**, 165–200.
20. Summers, M. D. & Anderson, D. L. (1973) *J. Virol.* **12**, 1336–1346.
21. Burton, A. & Sinsheimer, R. L. (1965) *J. Mol. Biol.* **14**, 327–347.
22. Kok, I. P., Chistyakova-Ryndich, A. V. & Gudz-Gorban, A. P. (1972) *Mol. Biol. USSR* **6**, 323–331.
23. Scharnhorst, D. W., Saving, K. L., Vuturo, S. B., Cooke, P. B. & Weaver, R. F. (1977) *J. Virol.* **21**, 292–300.
24. Yamafugi, K., Hashinaga, F. & Fujii, T. (1966) *Enzymologia* **31**, 92–104.
25. Kok, I. P., Chistyakova, A. V., Gudz-Gorban, A. P. & Solomko, A. P. (1968) *13th Int. Congr. Entomol. (Moscow)*, Abstr. Papers, p. 127.
26. Onodera, K., Komano, T., Himeno, M. & Sakai, F. (1965) *J. Mol. Biol.* **13**, 532–539.
27. Zherebstova, E. N., Strokovskaya, L. I. & Gudz-Gorban, A. P. (1972) *Acta Virol.* **16**, 427–431.